An unbalanced monocyte polarisation in peripheral blood and bone marrow of patients with type 2 diabetes has an impact on microangiopathy

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Abstract: AIM/HYPOTHESIS: Monocytes/macrophages play important roles in adipose and vascular tissues and can be polarised as inflammatory M1 or anti-inflammatory M2. We sought to analyse monocyte polarisation status in type 2 diabetes, which is characterised by chronic inflammation. METHODS: We enrolled 60 individuals without diabetes and 53 patients with type 2 diabetes. We quantified standard monocyte subsets defined by cluster of differentiation (CD)14 and CD16. In addition, based on the phenotype of polarised macrophages in vitro, we characterised and quantified more definite M1 (CD68(+)CCR2(+)) and M2 (CX3CR1(+)CD206(+)/CD163(+)) monocytes. We also analysed bone marrow (BM) samples and the effects of granulocyte-colony stimulating factor (G-CSF) stimulation in diabetic and control individuals. RESULTS: We found no alterations in standard monocyte subsets (classical, intermediate and non-classical) when comparing groups. For validation of M1 and M2 phenotypes, we observed that M2 were enriched in non-classical monocytes and had lower TNF- content, higher LDL scavenging and lower transendothelial migratory capacity than M1. Diabetic patients displayed an imbalanced M1/M2 ratio compared with the control group, attributable to a reduction in M2. The M1/M2 ratio was directly correlated with waist circumference and HbA1c and, among diabetic patients, M2 reduction and M1/M2 increase were associated with microangiopathy. A decrease in M2 was also found in the BM from diabetic patients, with a relative M2 excess compared with the bloodstream. BM stimulation with G-CSF mobilised M2 macrophages in diabetic but not in healthy individuals. CONCLUSIONS/INTERPRETATION: We show that type 2 diabetes markedly reduces anti-inflammatory M2 monocytes through a dysregulation in bone-marrow function. This defect may have a negative impact on microangiopathy.

DOI: https://doi.org/10.1007/s00125-013-2918-9

An unbalanced monocyte polarization in peripheral blood and bone marrow of type 2 diabetic patients impacts on microangiopathy

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Word count abstract 249; main text 3996.

ABSTRACT

Aim/hypothesis. Monocytes/macrophages play important roles in the adipose and vascular tissues and can be polarized as inflammatory M1 or anti-inflammatory M2. We sought to analyze monocyte polarization status in type 2 diabetes, which is characterized by chronic inflammation.

Methods. We enrolled 60 non diabetic individuals and 53 type 2 diabetic patients. We quantified standard monocyte subsets defined by CD14 and CD16. Additionally, based on the phenotype of polarized macrophages in vitro, we characterized and quantified more definite M1 (CD68+CCR2+) and M2 (CX3CR1+CD206+/CD163+) monocytes. We also analysed bone marrow (BM) samples and the effects of G-CSF stimulation in type 2 diabetic and control subjects.

Results. We found no alterations in standard monocyte subsets (classical, intermediate, and nonclassical) between groups. For validation of M1 and M2 phenotypes, we show that M2 were enriched in nonclassical monocytes, had lower TNF-α content, higher LDL scavenging and lower transendothelial migratory capacity than M1. Diabetic patients displayed an imbalanced M1/M2 ratio compared to controls, which was attributable to a reduction in M2. The M1/M2 ratio was directly correlated with waist circumference and HbA_{1c} and, among diabetic patients, M2 reduction and M1/M2 increase were associated with microangiopathy. A decrease in M2 was also found in the diabetic BM, with a relative M2 excess compared to the bloodstream. BM stimulation with G-CSF mobilized M2 in diabetic but not in healthy subjects.

Conclusion. We show that type 2 diabetes markedly reduces anti-inflammatory M2 monocytes, through a dysregulation in bone marrow function. This defect may negatively impact on microangiopathy.

Keywords: immune cells; inflammation; complications; obesity.

ABBREVIATION LIST

APC	Allophycocyanin
BM	Bone marrow
CCR2	Chemokine-C Receptor-2
CD	Cluster of Differentiation
CV	Coefficient of variation
CX3CR1	Chemokine-X3C Receptor-1
ESM	Electronic supplementary material
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony stimulation factor
HAEC	Human aortic endothelial cells
IFN	Interferon
IL	Interleukin
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PE	Phycoerithrin
TNF-a	Tumor necrosis factor-alpha

INTRODUCTION

Metabolic diseases are characterized by chronic systemic inflammation, but the mechanisms involved are unclear [1, 2]. In response to dietary challenges, adipocytes and endothelial cells activate classical inflammatory pathways, which impair the metabolic action of insulin, paving the way to diabetes [3]. In turn, hyperglycemia and hyperinsulinemia elicit multiple pro-inflammatory responses [4-6]. This is reflected by mild elevation of inflammatory markers in patients with diabetes or the metabolic syndrome [7]. Inflammation also promotes development and progression of diabetic complications, including atherogenesis, nephropathy, retinopathy, and neuropathy [8-11].

One pivotal aspect of innate immunity and chronic inflammation is the polarization of monocyte/macrophages, which are endowed of remarkable plasticity [12]. Tissue macrophages exist in 2 major states: classically activated inflammatory M1, and alternatively activated anti-inflammatory M2 [13]. Roughly, M1 express the macrophage marker CD68 and the chemokine receptor CCR2, while M2 typically express the macrophage marker CX3CR1 and scavenger receptors CD206 and CD163 [14]. It has been shown that M1 and M2 exert different actions in obesity and atherosclerosis [14, 15]. For instance, adipose tissue macrophages display a prevalent M1 phenotype that sustains inflammation and can be shifted toward M2 after weight loss [16]. In atherosclerosis, M1 are prone to become foam cells, while M2 exert scavenger activity and suppress inflammation [17]. To a similar extent, circulating monocytes are believed to reflect different inflammatory states. Based on CD14 and CD16 expression, the standard monocyte nomenclature distinguishes classical (CD14++CD16-), intermediate (CD14++CD16+) and non classical (CD14+CD16+) cells, with distinctive gene expression profiles [18, 19].

To date, the polarization status of circulating monocytes in type 2 diabetes is poorly characterized, and mainly derived from gene expression analyses [20, 21]. In this study, we aimed to determine the pro- versus anti-inflammatory monocyte polarization balance in type 2 diabetic patients compared to control subjects by identifying, characterizing and quantifying novel discrete cell populations.

MATERIALS AND METHODS

Patients. The protocol was approved by the local ethical committee and conducted in accordance to the Declaration of Helsinki as revised in 2000. Diabetic and control subjects were recruited at the Division of Metabolic Diseases of the University Hospital of Padova. All consecutive patients were deemed eligible pending they provided informed consent and satisfied inclusion/exclusion criteria. Type 2 diabetes was defined according to ADA criteria [22]. Control subjects had normal fasting plasma glucose and HbA_{1c} (IFCC-aligned HPLC method)<6.0% (<42 mmol/mol). Subjects aged 25-80 could be included; exclusion criteria were: acute disease or infection, immunosuppression/organ transplantation, chronic inflammatory diseases, recent surgery, trauma or cardiovascular events, pregnancy/lactation. For all patients, we recorded the following data: age, sex, BMI, waist circumference, blood pressure, history of hypertension, smoking, lipid profile, fasting plasma glucose, HbA_{1c}, creatinine. Diabetic retinopathy was diagnosed by digital funduscopy. Diabetic nephropathy was defined as an urinary albumin/creatinine ratio >30 mg/g or an estimated glomerular filtration rate (eGFR) <60 ml min^{-1} 1.73 m^{-2}. Coronary artery disease was defined as a history of myocardial infarction or angina, confirmed by coronary angiography or a myocardial stress test. Peripheral arterial disease was defined as a history of claudicatio or rest pain with evidence of leg artery stenosis at invasive or non invasive examinations. Cerebrovascular disease was defined as a history of stroke or evidence of >30% carotid stenosis at ultrasound examination. Atherosclerosis/macroangiopathy were defined as the presence of either coronary, peripheral or cerebrovascular disease. We also collected data on medications.

Bone marrow samples. Patients with heart disease, with (n=5) and without (n=5) type 2 diabetes, recruited at the Johann Wolfgang Goethe University Hospitals for cell therapy were included. The ethics review board of Goethe University approved the protocols (NCT00962364 and NCT00284713). After giving informed consent, before cell therapy, patients were subjected to bone marrow (BM) aspiration and about 1 ml of the BM aspirate was collected for M1/M2 quantification.

Bone marrow stimulation. In a prospective clinical trial (NCT01102699), we enrolled 13 type 2 diabetic and 14 non diabetic subjects, who underwent BM stimulation with a single subcutaneous injection of 5 μg/kg Filgrastim (Granulokine, Amgen, Thousand Oaks, CA, USA) [23]. Before and 24h after injection, blood samples were drawn determination of M1/M2.

Identification and characterization of monocyte subsets. Identification of monocyte subsets was performed using multi-parameter flow cytometry. For analysis of classical, intermediate, and non classical monocytes, cells were stained with a FITC or PE anti-CD14 mAb (BD Pharmingen, Franklin Lakes, NJ USA) and a FITC- or PE-Cy5 anti-CD16 mAb (Beckman Coulter, Pasadena, Ca, USA). The analysis was performed according to standardized gating strategy [18]. For more definite monocyte subsets, we stained with FITC anti-CD68 mAb (Dako, Milan, Italy) and PE or AlexaFluor-647 anti-CCR2 mAb (R&D Systems, Minneapolis, MN, USA) for identification of M1 cells and with FITC anti-CX3CR1 (Biolegend), PE anti-CD163 (BD) and APC anti-CD206 (BD) mAbs for M2. M1 were defined as CD68+CCR2+ cells and M2 were defined as CX3CR1+CD163+/CD206+. In preliminary experiments, we found a strong correlation between CD163 and CD206 staining on CX3CR1+ cells, suggesting that expression of these scavenger receptors on monocytes convey similar information. Thus, M2 were defined as CX3CR1+ cells that express either CD163 or CD206. The relative frequency of these monocyte subsets were expressed as % of the total monocyte gate. Reproducibility of M1 and M2, assessed as coefficient of variation in a test-retest sample of 10 subjects was 7.8% and 8.9%, respectively.

In separate experiments performed with peripheral blood of healthy subjects, M1 and M2 cells were stained with CD14 and CD16. Gated CD68+CCR2+ (M1) and CX3CR1+CD206+ (M2) cells were tested against standard monocyte subsets, allowing the positioning of M1 and M2 cells within the traditional CD14/CD16 monocyte subsets.

For the study of phagocytosis of modified LDL particles, M1 (CD68+CCR2+) and M2 (CX3CR1+CD206+) cells were incubated with 10 μg/ml DiI-labeled acetylated LDL (DiI-AcLDL, Molecular Probes, Eugene, OR, USA) for 2 hours at 37°C. The percentages of cells positive for DiI-AcLDL in the M1 or M2 gate were analyzed.

We also quantified the expression of pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines on M1 and M2 cells. To this aim, cells were stained with M1 or M2 markers, permeabilized and stained with APC-conjugated anti-human TNF-α or IL-10 (BD) after 2h incubation with 2 μM monensin. The percentages of M1 (CD68+CCR2+) or M2 (CX3CR1+CD163+) cells expressing TNF-α or IL-10 were calculated.
M1 (CD68+CCR2+) and M2 (CX3CR1+CD163+) cells were also analyzed for expression of the G-CSF receptor CD114 using an APC-conjugated anti-CD114 mAb (BD).

Primary culture of polarized macrophages. Venous blood was obtained from healthy donors and separated using a Ficoll-Paque solution. Mononuclear cells were collected, washed with PBS containing EDTA (5 mM) and resuspended at 2-3×10^6 cells/ml in RPMI-1640 supplemented with glutamine, penicillin-streptomycin and 15% FCS. Monocytes were separated from lymphocytes by adherence to 100-mm plastic dishes for 2 hours. Adherent monocytes were cultured in fresh medium for 7 days at 37°C to allow spontaneous differentiation into macrophages. Then, resting cells were polarized into M1 or M2 macrophages by 48h incubation with LPS (1 μg/ml) and IFN-γ (10 ng/ml) or IL-4 (20 ng/ml) and IL-13 (5 ng/ml), respectively. In separate experiments, M1 and M2 stimuli were added since the beginning of the culture, for 7 days.

Ex vivo stimulation of monocytes. Peripheral blood mononuclear cells were obtained as described above. Cells were incubated for 3 hours at 37°C, then non-adherent cells were discarded and the remaining adherent fraction (monocytes) was incubated with 20 ng/ml of recombinant G-CSF for 24h. Control cells where incubated with equal volume of PBS. After treatment, cells were washed twice with PBS to remove non adherent cells and detached for analysis.

Trans-endothelial migration. To test the ability of M1 and M2 cells to undergo transendothelial migration, we used a modified transwell migration assay [24]. Freshly isolated human PBMC were left to adhere to or transmigrate through a monolayer of red PKH26-labeled (Sigma Aldrich, St. Louis, MO, USA) human aortic endothelial cells grown to confluence on the filter membrane of sterile 3 micron-pore, 12-well format transwell migration inserts (BD). After 6h, we collected transmigrating PBMC from the lower compartment

(migrated fraction) and adherent cell population by sequential washing/detachment. The 2 cell fractions (adherent and migrated) were stained with M1 (CD68+CCR2+) and M2 (CX3CR1+CD206+) markers to look for enrichment of M1 and M2 cells in the migrated versus adherent fraction. Endothelial cells were separated from PBMC during the analysis by a multiparametric gating strategy.

Statistical analyses. Data are expressed as mean±standard error or as percentage. Normality of the variables of interest were checked with the Kolmogorov-Smirnov test. Non normal variables were log-transformed for analyses. Comparisons between 2 groups were performed using Student's t test for normal variables and with Mann-Whitney's U test for non normal variables. Percentages were compared using the Chi-square test. Multiple linear regression analyses were performed entering cell levels as the dependent variable and clinical characteristics as independent variables in a single block. Explanatory variables were selected as those that were significantly different upon univariate group comparison in table 1 plus gender, but medications were not included to avoid overfitting. Statistical significance was accepted at $p<0.05$; SPSS ver. 18 was used.

RESULTS

Standard CD14/CD16 monocyte subsets in diabetic patients and control subjects.

In 60 controls with normal glucose metabolism and 53 type 2 diabetic patients, we determined the percentages of classical (CD14++CD16-), intermediate (CD14++CD16+) and non-classical (CD14+CD16+) monocytes (Fig. 1a,b), as well as of M1 (CD68+CCR2+) and M2 (CX3CR1+CD163+/CD206+) cells. Clinical characteristics are reported in Table 1. In control subjects, classical, intermediate and non-classical monocytes accounted for 32.4±4.4%, 45.4±4.3% and 7.8±0.6%, respectively. We found no significant differences in these monocyte subsets in type 2 diabetic patients versus controls (Fig. 1c).

Functional characteristics of circulating M1 and M2 monocytes.

Based on the negative results of the analysis of standard CD14/CD16 monocyte subsets, we moved to analyze cells expressing more definite M1 (CD68 and CCR2) or M2 (CX3CR1, CD163 and CD206) markers (Fig. 1d,e). We compared these circulating cells with monocyte-macrophage phenotypes and performed a functional characterization for validation.

Analogy to in vitro polarized macrophages. We first determined whether M1 and M2 markers used here to define monocytes are expressed by cultured macrophages polarized in vitro with standard protocols. M1 cells obtained after incubating monocytes of healthy donors with LPS and INF-γ showed a 3.6±1.2 fold increase in CD68, a 74.9±8.5% reduction of CD206 and a 87.4±5.8% reduction in CD163 expression compared to unstimulated cells, while CCR2 did not significantly change. In M2 cells obtained by stimulation with IL-4+IL-13, CD206 and CD163 expression was 24.2±8.4 and 16.4±5.0 fold higher, respectively, than in M1 cells. M2 markers were increased after a 7-day stimulation compared to unstimulated cells. (ESM Fig. 1a). These data indicate that the M1 and M2 markers used to define monocyte subsets resemble macrophage phenotypes in vitro, with the exception of chemokine receptors CCR2 and CX3CR1.

Comparison of monocyte-macrophage gene expression between M1 and M2 cells and in vitro polarized macrophages was also performed *in silico* using public resources (GEO accession number GSE5099 [25]). While the chemokine receptor genes CCR2 and CX3CR1 are rapidly downregulated during monocyte-

macrophage differentiation, CD206 and CD16 are over-expressed in M2 compared to M1 macrophages and can be considered genes distinctive of the M2 phenotype both in the blood and in vitro (ESM Fig. 2a).

Comparison with CD14/CD16 monocytes. To further clarify the identity of M1 and M2 monocytes defined by the selected antigenic profiles, we stained CD68+CCR2+ (M1) and CX3CR1+CD206+ (M2) cells with CD14 and CD16, which allows a standardized definition of monocyte subsets. We found that percentage of cells belonging to the classical CD14++CD16- monocytic fraction was 2-fold higher for M1 compared to M2 (22.8±2.3% vs 11.3±4.2%; p=0.05), while the percentage of cells belonging to nonclassical CD14+CD16+ monocytes was 4-fold higher for M2 compared with M1 (8.4±1.8% vs 2.0±0.8%; p=0.03) (ESM Fig. 1b). These data indicate that M1 and M2 cells have different monocytic features, and support the definition of CD68+CCR2+ cells as classically inflammatory monocytes and CXC3CR1+CD206+ cells as anti-inflammatory cells [26]. The analysis of gene expression from public resources (GEO accession number GSE25913 [19]) confirms that CX3CR1 is about 2.2-fold over-expressed in CD14++CD16+ compared to CD14++CD16- monocytes, while CD163 appears to be more expressed in CD16- compared to CD16+ cells (ESM Fig. 2b). These data suggest that the analysis of surface protein expression by flow cytometry provides complementary information over and beyond gene expression.

Cytokine expression. We analyzed the expression of TNF-α and IL-10 by intracellular flow cytometry. We found that 8.5±2.9% of M1 (CD68+CCR2+) and 2.0±0.7% of M2 (CX3CR1+CD163+) were brightly positive for TNF-α ($p<0.05$), while the expression of IL-10 was not significantly different (M1 7.6±3.0% vs M2 5.6±1.1%; p=0.72) (ESM Fig. 1c).

Uptake of modified LDL. After staining peripheral blood cells with DiI-labeled acetylated LDL, we found that about 5% of circulating monocytes took up AcLDL while lymphocytes were negative for DiI-AcLDL uptake, indicating that a fraction of monocytes has phagocytosis activity. We found that CX3CR1+CD206+ M2 cells showed a 40.8±14.3% increased LDL uptake compared to CD68+CCR2+ M1 cells (p=0.02) (ESM Fig. 1d). This is consistent with the notion that alternatively activated macrophages (M2) have a distinctive phagocytic activity (28), possibly related to the higher expression of scavenger receptors.

Transendothelial migration. Spontaneous migration across an endothelial layer as well as MCP-1 or fractalkine directed migration was studied and the ratios between the percentages of cells staining for M1 or M2 markers in the migrated versus the adherent compartment were compared. This ratio was significantly

higher for M1 than for M2 cells (ESM Fig. 1e), indicating that M1 cells have a higher spontaneous- and chemokine-induced transendothelial migratory capacity than M2. The amount of cells migrated toward chemokines was underestimated owing to chemokine receptor (CCR2 and CX3CR1) internalization during ligand stimulation. Therefore, that M1 migrated more than M2 independently of the agonist used, points to an intrinsically higher migratory capacity of M1 than M2.

Circulating M1/M2 polarization balance in diabetes.

The validation analysis indicates that circulating M1 and M2 cells share characteristics with *in vitro* polarized macrophages and have different functions. Therefore, we quantified these monocyte phenotypes in type 2 diabetic patients versus healthy controls.

In healthy controls, 38.0±3.0% of circulating monocytes had a M1 phenotype and 20.2±2.3% satisfied M2 criteria. Thus, the normal M1/M2 polarization balance (M1/M2 ratio) was 1.9. The remaining ~40% of monocytes did not satisfy neither M1 nor M2 criteria, indicating they are less differentiated. Type 2 diabetic patients showed a striking reduction of M2 (7.7±1.6%; $p=4x10^{-5}$) with no change in M1 (36.9±2.9%; p=0.79; Fig. 1f). As a result, the M1/M2 ratio was markedly increased in type 2 diabetes (4.8±0.8; $p<0.001$) (Fig. 1g). Diabetic patients were older and had a worse risk profile than controls, but the decrease in M2 remained significantly associated with diabetes upon correction for confounders (ESM Table 1). We also analyzed separately 10 drug-naïve diabetic patients with a shorter disease duration (3.2±1.4 vs 12.3±3.8 years; $p<0.01$), and found that M1 and M2 levels, as well as the M1/M2 ratio were similar to the rest of the diabetic population (Fig. 1h,i).

These data indicate that, while the traditional monocyte nomenclature is poorly informative, type 2 diabetes affects the pattern of M1/M2 monocyte polarization, which is characterized by M2 deficiency.

Relationships with clinical characteristics and complications.

In the whole population, we found that the M1/M2 ratio was correlated with waist circumference (r=0.22; p=0.018) and HbA_{1c} (r=0.26; p=0.005). We then looked for associations between types and severity of complications and alterations in monocyte subtypes. While M1 cells showed no consistent trend in relation to complications (Fig. 2a), we found a trend toward decreased M2 in patients with nephropathy or

retinopathy (Fig. 2b). The M1/M2 ratio was significantly reduced in patients with nephropathy and a trend was detected for retinopathy (Fig. 2c). When patients were divided according to the presence of microangiopathy or macroangiopathy or both, M2 levels and the M1/M2 appeared to be related to microangiopathy (Fig. 2d-f).

Monocyte phenotypes in the diabetic bone marrow.

We then examined the percentages of M1 and M2 cells in BM aspirates of 5 type 2 diabetic and 5 control individuals (ESM Table 2). In control subjects, M1/M2 polarization in the bone marrow was inverted compared to peripheral blood, with a predominance of M2 (29.3±4.0%) versus M1 (19.6±6.2%) (Fig. 3a). Both M1 (10.9±2.0%: p=0.23) and M2 (16.4±2.0%; p=0.029) were reduced in the diabetic bone marrow but only M2 reduction was statistically significant. The BM M1/M2 polarization balance was identical between diabetic and control subjects (Fig. 3b).

Despite M2 reduction in the diabetic BM, M2 percentages were much higher than in peripheral blood (PB). As monocytes are mobilized from the BM to the PB, we determined the BM/PB ratio in type 2 diabetic and control patients, as an indicator of the gradient between these 2 compartments. Compared to controls, diabetic patients showed a relative excess of M2 cells in the bone marrow (Fig. 3c).

G-CSF mobilizes bone marrow M2 in diabetes.

Given the different M1 and M2 levels in the BM of type 2 diabetic patients compared to controls, we quantified circulating M1 and M2 cells in diabetic patients and controls undergoing BM stimulation with G-CSF in the clinical trial NCT01102699 (ESM Table 3). Twenty-four hours after G-CSF injection, the M1/M2 remained stable in controls (Fig. 3d) and dropped in type 2 diabetic patients (Fig. 3e). This was attributable to an increase in M2 after G-CSF only in diabetic patients. These data indicate that G-CSF reverses the M1/M2 alterations in diabetes and indirectly confirm the relative excess of M2 in the BM. To understand why G-CSF mobilizes preferentially M2 cells, we analyzed the surface expression of the G-CSF receptor CD114 and found that 85.2±2.2% of M1 (CD68+CCR2+) and 83.6±3.1% of M2 (CX3CR1+CD163+) expressed CD114 (p=0.39) (ESM Fig. 3a). This rules out that different G-CSF receptor expression on M1 and M2 cells accounts for the differential effect of G-CSF on M2 versus M1 mobilization. To rule out that

the effect of G-CSF on the M1/M2 balance was due to direct polarization rather than mobilization, we treated *ex vivo* monocytes from type 2 diabetic or control subjects for 24 hours with the peak concentrations of G-CSF that are achieved *in vivo* after 5 mcg/kg injection (i.e. 20 ng/ml), and found no significant effect of G-CSF on the M1/M2 ratio (ESM Fig. 3b). These results are consistent with the notion that G-CSF does not directly affect function of the cells that are mobilized [27].

DISCUSSION

By studying novel phenotypes of monocyte polarization, we show that type 2 diabetes is associated with a marked reduction of the anti-inflammatory M2 phenotype. Traditionally, monocyte subsets are defined by the expression of CD14 (LPS receptor) and CD16 (FcγRIII) [18]. Previous studies reported changes in CD14/CD16 expressing monocytes in relation to obesity and weight loss [28], but the effect of diabetes is controversial [29, 30]. With this standard nomenclature, we found no significant alteration in classical CD14++CD16-, intermediate CD14++CD16+ and nonclassical CD14+CD16+ monocytes in type 2 diabetic patients versus controls. Specific characteristics of the cohorts under investigation may account for partial inconsistency with the literature. In addition, this definition does not distinguish between M1 and M2 cells. We therefore analyzed more definite monocyte polarization phenotypes, using antigenic profiles derived from studies on the monocyte-macrophage differentiation cascade [15] in order to identify circulating cells more closely resembling tissue macrophages. Cells co-expressing CD68 (a LDL- and lectin-binding scavenger protein) and CCR2 (MCP-1 receptor) were considered pro-inflammatory M1 cells, while cells co-expressing CX3CR1 (fractalkine receptor) and the scavenger receptors CD163 or CD206 were considered anti-inflammatory M2 [14]. Based on these antigenic profiles, M1 are expected to enter the diseased vascular wall following MCP-1 gradients and to become foam cells [17], while M2 should patrol the vasculature and clean up the tissue via scavenger activity [14]. We performed a series of experiments to validate these phenotypes: M2 cells, compared to M1, were 4-fold enriched in nonclassical monocytes involved in the resolution inflammation, and showed lower TNF-α content and higher capacity of cleaning acetylated LDL. Moreover, the circulating M2 phenotype resembled in vitro polarized M2 macrophages, which were CX3CR1+CD163+CD206+. Finally, M1 cells had a much stronger capacity of transendothelial migration than M2, suggesting they are more prone to enter the diseased vessel wall. Therefore, the selected M1 and M2 monocyte phenotypes truly reflect cells with different inflammatory potential and resemble the corresponding macrophage populations.

We found that type 2 diabetes is characterized by a marked reduction in M2 cells while M1 were unchanged compared to controls; as a result, the M1/M2 polarization ratio was increased in diabetes. As type 2 diabetes is considered a pro-inflammatory condition, it is striking that the monocyte polarization imbalance is

attributable to a defect in anti-inflammatory cells, rather than an excess of pro-inflammatory ones. This observation is however in line with the theory that diabetes is a disease of impaired damage control [31], in which injury is worsened by defective repair. The M1/M2 polarization ratio was directly correlated to waist circumference, an indicator of central obesity and insulin resistance, and to HbA_{1c}, an indicator of glucose control. In addition, the pattern of complications showed trend associations with altered polarization. The presence of microangiopathy, especially nephropathy, was associated with a reduction in M2 and an increase in the M1/M2 ratio. Despite previous studies indicate a prominent role of M1/M2 cells in atherosclerosis [14, 32], we found no association with diabetic macroangiopathy, but the relationships between M1/M2 and diabetic complications should be confirmed in a larger cohort. Experimental studies indicate that polarization of kidney macrophages is related to renal injury and function [33]. Only 2 diabetic patients had macroalbuminuria and all those with reduced eGFR had mild renal failure, thus preventing any correlation with the severity of nephropathy. Nonetheless, this is the first report of an association between diabetic nephropathy and altered monocyte polarization. The levels of intermediate CD14++CD16+ cells, to which most M1 and M2 cells belong, was shown to be predictive of cardiovascular outcomes in patients with chronic kidney disease [34]. As M1 and M2 are more detailed phenotypes than traditional monocyte subsets, the prognostic meaning of the M1/M2 ratio warrants investigation. Interestingly, decrease in M2 and increase in the M1/M2 ratio was already evident in drug-naïve patients with a short disease duration.

Recent data indicate that diabetes induces BM microangiopathy, with features similar to those seen in the kidney and in the retina [35-37]. In turn, microangiopathy causes BM dysfunction and alters stem cell regulation [23]. Thus, we analyzed monocyte subsets in BM aspirates of type 2 diabetic and control patients. First, it was noteworthy that the M1/M2 balance was inverted compared to PB, suggesting that monocyte subsets play a role in BM function [38]. In diabetes, M2 cells were significantly reduced compared to controls in the BM, but to a lesser extent than in the PB. Such a BM-to-PB gradient indicated a relative excess of M2 in the diabetic BM. This was confirmed by showing that G-CSF preferentially mobilizes M2 only in diabetic patients, restoring the M1/M2 balance toward normal levels. All patients of the BM substudy had heart disease, thus limiting generalizability of the findings to the general diabetic population. Notwithstanding this limitation, it appears that type 2 diabetes associates with a depletion of circulating M2, which are stuck in the BM and can be mobilized by pharmacologic G-CSF doses.

Reversal of M1/M2 imbalance in diabetes with BM stimulation indicates that BM dysfunction plays a role in diabetes-related inflammation and represents a possible link between distant end-organ complications. Speculatively, BM microangiopathy, by altering the monocyte polarization status, might contribute to the development or progression of microangiopathy in other organs (Fig. 4). As monocyte-macrophages are involved in vascular disease and regulate the cross-talks with inflammatory pathways [14], the M1/M2 imbalance in diabetes can exert a negative impact for the development of diabetic complications.

Thus, M1/M2 cells may be considered metabolic-inflammatory biomarkers of the overall risk in diabetic patients. Of particular interest is the modulation of M1/M2 polarization by lifestyle and anti-diabetic medications, particularly those with distinctive anti-inflammatory properties, such as glitazones and DPP-4 inhibitors. The prognostic meaning of the reduced M2 level in diabetic patients also warrants further investigation.

ACKNOWLEDGEMENTS

Author Contributions: conception and design (GPF, SVdK, AC, CA, AA); acquisition of data (GPF, SVdK, EB, MA, RC, NK, UL, AT, CB, FS, SD, AZ); analysis and interpretation of data (GPF, SVdK, EB, MA, RC, NK, UL); drafting the article (GPF, SVdK, AA); revising the article (EB, MA, RC, NK, UL, AT, CB, AC, FS, SD, AZ, CA); all authors provided final approval of the version to be published.

Financial support: GPF is supported by a European Foundation for the Study of Diabetes (EFSD)/Sanofi Clinical Investigator Fellowship.

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1. Analysis of traditional and novel monocyte subsets. a-c) In type 2 diabetic (T2D) and control (non DM) subjects, we quantified the traditional monocyte subsets by CD14 and CD16 staining. Representative scatter plots of a patient with predominance of classical CD14++CD16- monocytes (a) and of a patient with prevalence of intermediate CD14++CD16+ monocytes (b). c) Quantification of these phenotypes did not identify any significant difference between controls (white bars) and type 2 diabetes (black bars). d, e) The gating strategy used to identify and quantify circulating CD68+CCR2+ (M1) and CX3CR1+CD206+/CD163+ (M2) cells in a non diabetic (d) and a diabetic (e) patient. f) We quantified M1 (CD68+CCR2+) and M2 (CX3CR1+CD206+/CD163+) cells in control subjects (white bars) and type 2 diabetic patients (black bars). g) The M1/M2 ratio was calculated to represent the monocyte polarization balance. h, i) Comparison between drug-naïve T2D patients (n=10, white bars) and other T2D patients included in the study (n=43, black bars). *$p<0.05$ versus CTRL.

Fig. 2. **Levels of M1 and M2 and M1/M2 ratio in relation to diabetic complications**. a-c) Percentages of M1 monocytes (a), M2 monocytes (b), and the M1/M2 polarization ratio (c) in type 2 diabetic patients divided according to the absence (white bars) or presence (black bars) of diabetic complications. *$p<0.05$ versus absence of complication. d-f) Percentages of M1 monocytes (d), M2 monocytes (e), and the M1/M2 polarization ratio (f) in type 2 diabetic patients without complications (white bars), with macroangiopathy alone (light grey bars), with microangiopathy alone (dark grey bars) and with both micro- and macroangiopathy (black bars). *$p<0.05$ versus no complication. ATH, atherosclerosis.

Fig. 3. Bone marrow M1 and M2 cells. a) M1 (white bars) and M2 (black bars) cells were quantified in bone marrow samples of 5 controls and 5 type 2 diabetic patients. *$p<0.05$ versus controls. b) The M1/M2 ratio in the bone marrow was calculated for controls and type 2 diabetic patients. c) The BM to PB ratio, as an index of the gradient between these 2 compartments, was calculated for M1, M2, and M1/M2 ratio in controls (white bars) and type 2 diabetic patients (black bars). *$p<0.05$ versus controls. d) Pre- (white bars) and post- G-CSF (black bars) levels of M1 and M2 cells and the M1/M2 ratio (scale on the right) in control

subjects. e) Pre- (white bars) and post- G-CSF (black bars) levels of M1 and M2 cells and the M1/M2 ratio (scale on the right) in type 2 diabetic patients. *$p<0.05$ versus controls in (d); †$p<0.05$ versus pre-G-CSF levels.

Fig. 4. Pathophysiological model. A model of the cross-talk between microangiopathy in the bone marrow (BM) and in other organs through an imbalance in M1/M2 polarization is shown. Diabetes induces pathological alterations in the BM, which are similar to the microangiopathy observed in other organs, such as the kidney. BM alterations seem to account for the altered M1/M2 polarization status in peripheral blood. As M1/M2 imbalance is related to microangiopathy, especially nephropathy, it is tempting to speculate that BM and kidney microangiopathy are pathophysiologically related through dysregulation of BM-derived cells, such as M1 and M2 monocytes.

Table 1. Characteristics of the study population. *p<0.05 vs controls.

Variable	Controls	T2D
Number	60	53
Age, years	57.6±1.3	61.6±1.3*
Sex male, n (%)	31 (51.7)	38 (69.8)
BMI, kg m^{-2}	26.1±1.3	28.5±0.8*
Fasting plasma glucose, mmol/l	5.08±0.06	8.67±0.32*
HbA_{1c}, % (mmol/mol)	5.5±0.1 (36.6±0.7)	7.7±1.2 (60.7±9.5)*
Hypertension, n (%)	20 (33.9)	41 (77.3)*
Systolic blood pressure, mm Hg	134.0±2.5	139.2±2.6
Diastolic blood pressure, mm Hg	84.8±1.4	81.3±1.3
Total cholesterol, mmol/l	5.21±0.16	4.41±0.13*
HDL cholesterol, mmol/l	1.52±0.06	1.26±0.06*
LDL cholesterol, mmol/l	3.04±0.13	2.49±0.1*
Triglycerides, mmol/l	1.42±0.13	1.44±0.1
Albumin excretion rate, mg g^{-1} creatinine	3.0±0.9	151.2±91.4*
Serum creatinine, µmol/l	81.8±3.6	90.9±4.8
eGFR < 60 ml min^{-1} 1.73 m^{-2}, n (%)	0 (0.0)	6 (11.3)*
Retinopathy, n (%)	0 (0.0)	11 (20.7)*
Coronary artery disease, n (%)	2 (3.3)	12 (22.6)*
Atherosclerotic CVD, n (%)	5 (8.3)	27 (50.9)*
Insulin, n (%)	0 (0.0)	35 (66.0)*
Metformin, n (%)	0 (0.0)	36 (67.9)*
Secretagogues, n (%)	0 (0.0)	23 (43.4)*
Incretin drugs, n (%)	0 (0.0)	4 (7.5)*
ACEi / ARBs, n (%)	17 (28.3)	40 (75.5)*
Other hypotensives, n (%)	10 (16.7)	30 (56.6)*
Anti-aggregants, n (%)	11 (18.3)	30 (56.6)*
Statin, n (%)	32 (53.3)	33 (62.2)

Fig. 1

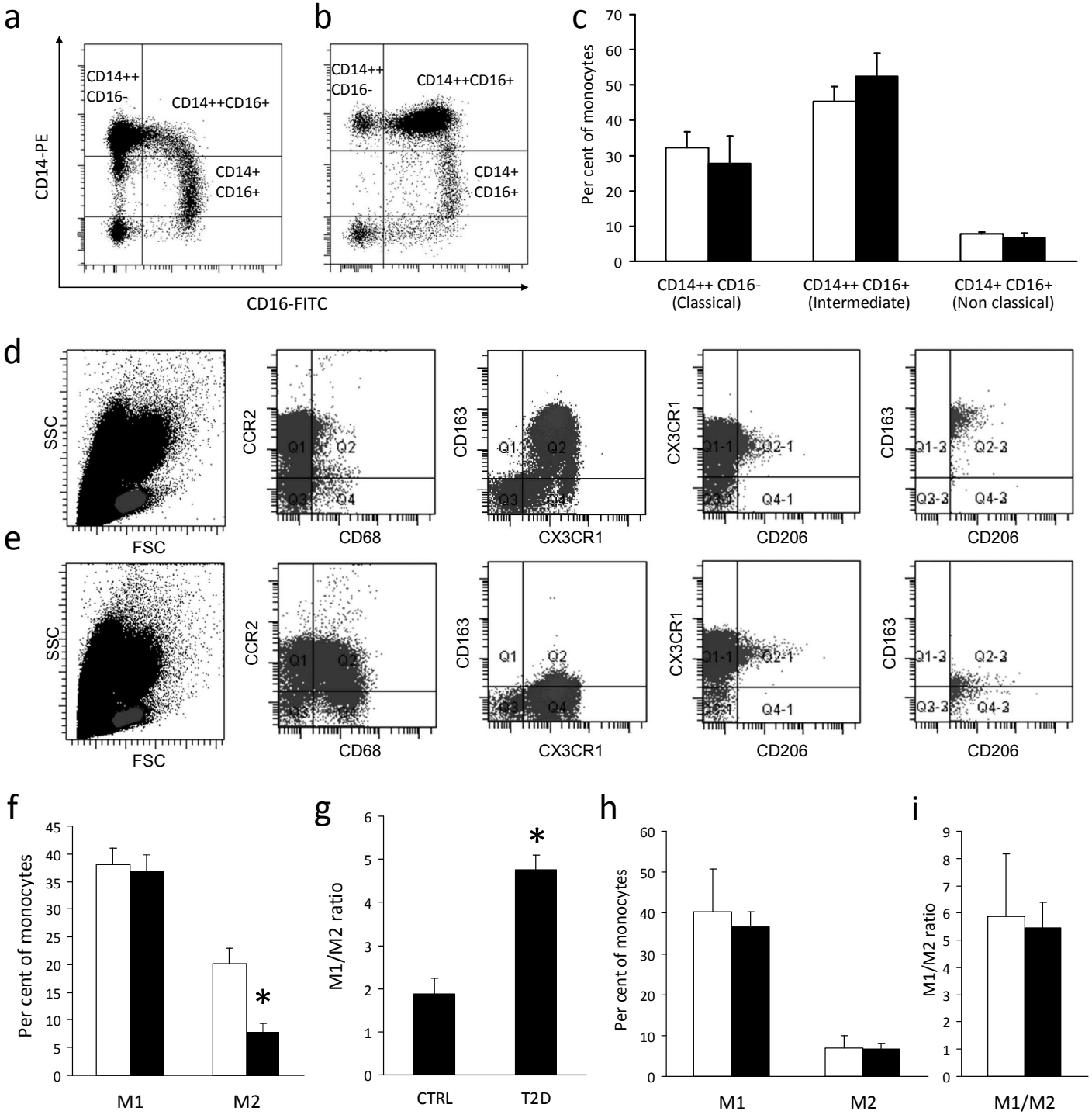

a
b
c
CD14++ CD16-
CD14++CD16+
CD14+ CD16+
CD14-PE
CD16-FITC
Per cent of monocytes
CD14++ CD16- (Classical)
CD14++ CD16+ (Intermediate)
CD14+ CD16+ (Non classical)
d
e
SSC
FSC
CCR2
CD68
CD163
CX3CR1
CD206
Q1
Q2
Q3
Q4
Q1-1
Q2-1
Q4-1
Q1-3
Q2-3
Q3-3
Q4-3
f
g
h
i
Per cent of monocytes
M1/M2 ratio
M1
M2
CTRL
T2D
M1/M2
*

Fig. 2

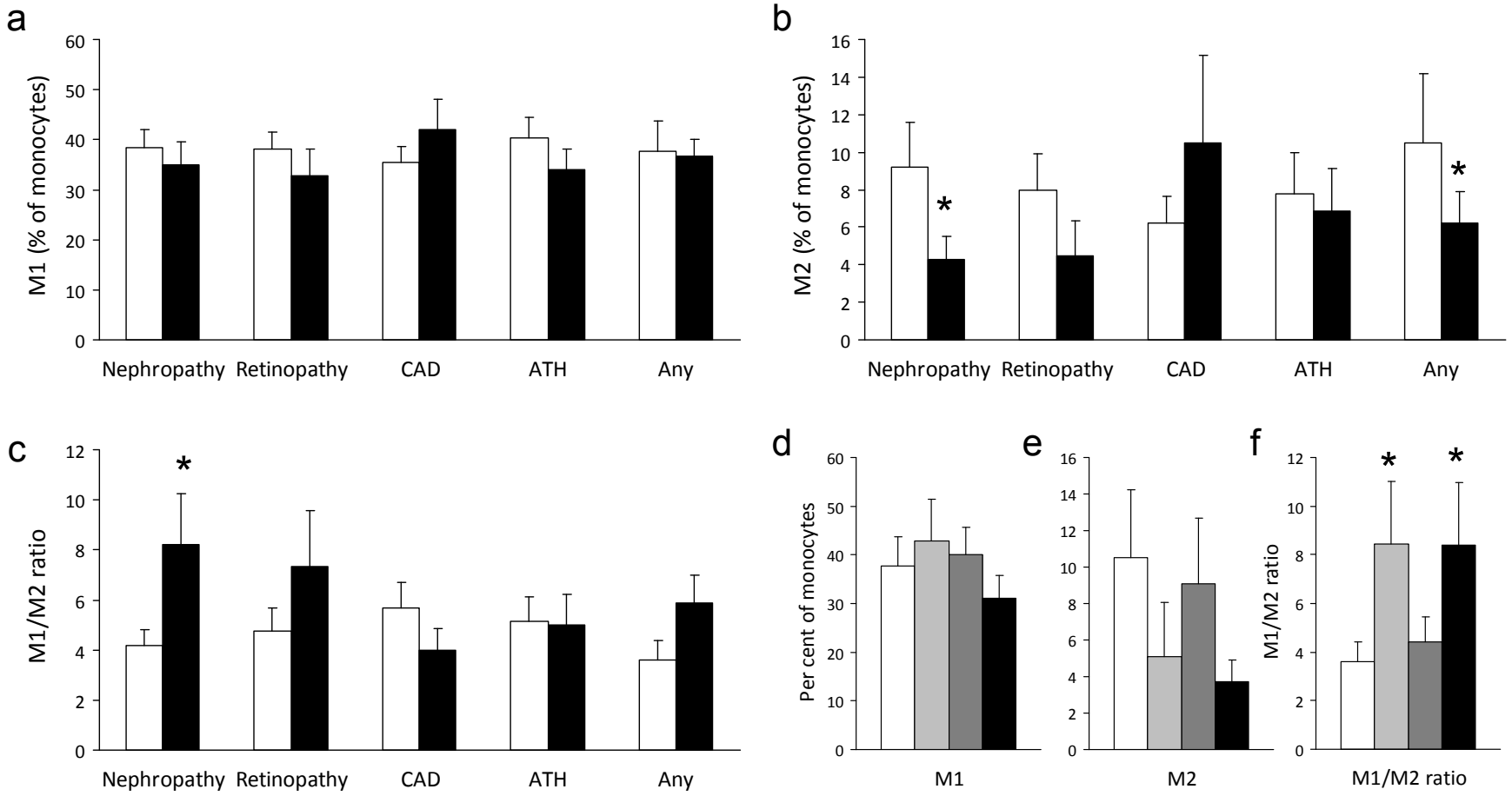

a
M1 (% of monocytes)
60
50
40
30
20
10
0
Nephropathy
Retinopathy
CAD
ATH
Any
b
M2 (% of monocytes)
16
14
12
10
8
6
4
2
0
*
*
Nephropathy
Retinopathy
CAD
ATH
Any
c
M1/M2 ratio
12
10
8
6
4
2
0
*
Nephropathy
Retinopathy
CAD
ATH
Any
d
Per cent of monocytes
60
50
40
30
20
10
0
M1
e
16
14
12
10
8
6
4
2
0
M2
f
M1/M2 ratio
12
10
8
6
4
2
0
*
*
M1/M2 ratio

Fig. 3

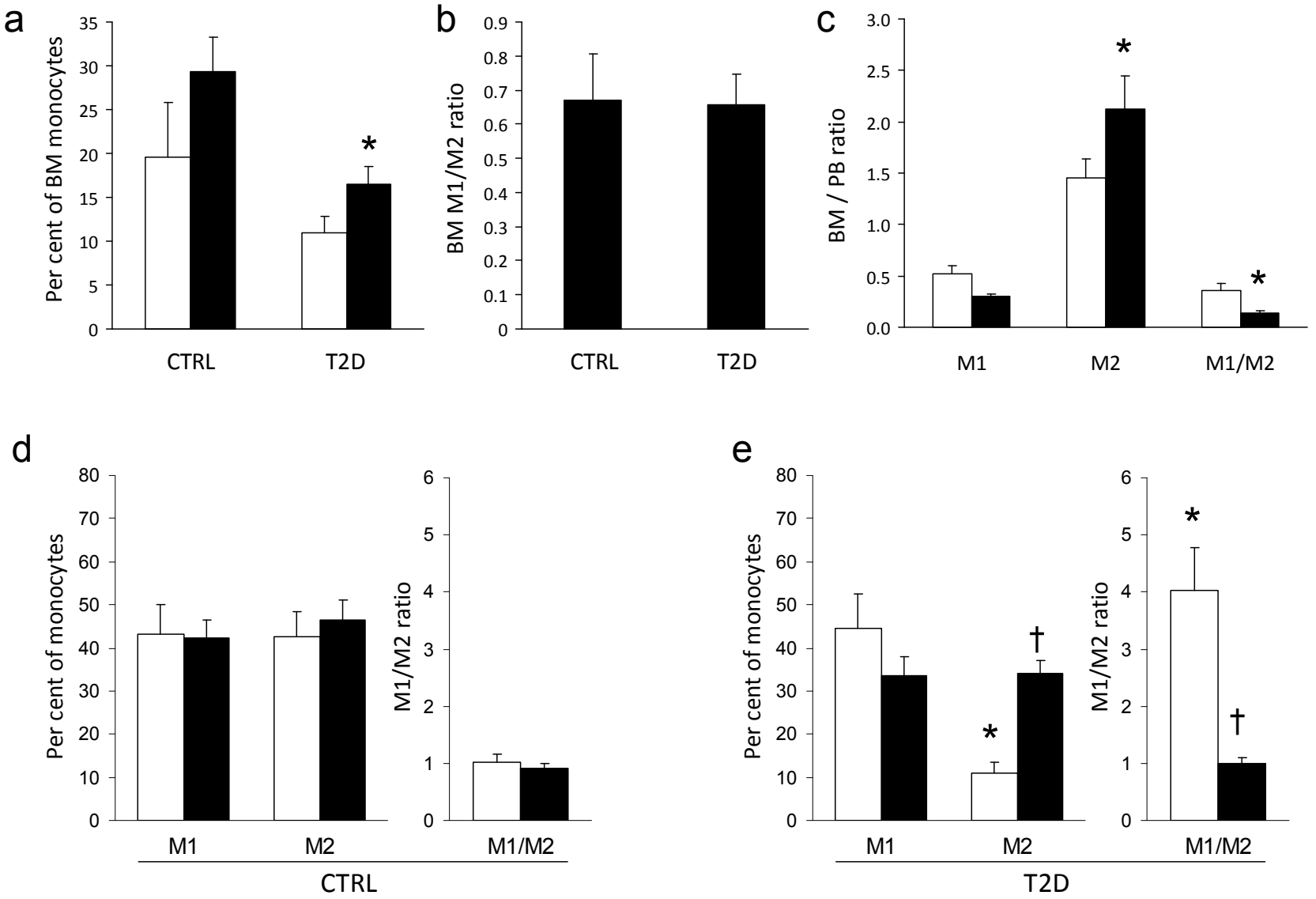
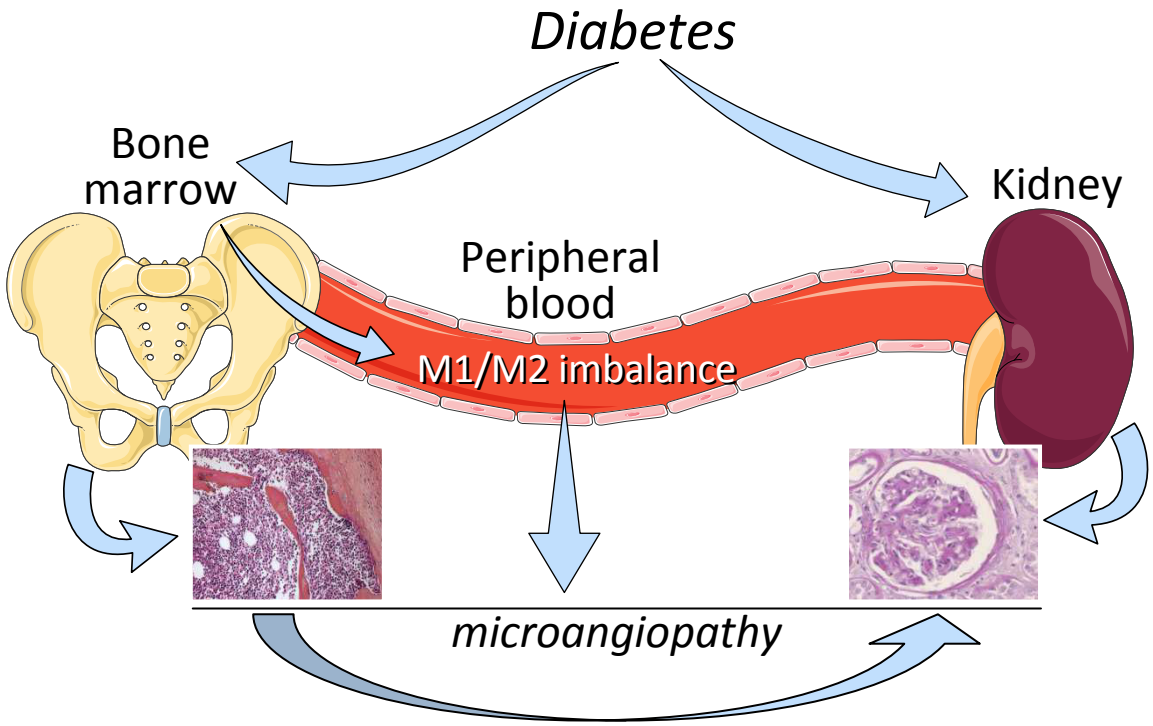

a
Per cent of BM monocytes
35
30
25
20
15
10
5
0
*
CTRL
T2D
b
BM M1/M2 ratio
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CTRL
T2D
c
BM / PB ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
M1
M2
M1/M2
d
Per cent of monocytes
80
70
60
50
40
30
20
10
0
M1/M2 ratio
6
5
4
3
2
1
0
M1
M2
M1/M2
CTRL
e
Per cent of monocytes
80
70
60
50
40
30
20
10
0
*
†
M1/M2 ratio
6
5
4
3
2
1
0
*
†
M1
M2
M1/M2
T2D

Fig. 4


Diabetes
Bone marrow
Kidney
Peripheral blood
M1/M2 imbalance
microangiopathy

Unbalanced monocyte polarization in peripheral blood and bone marrow of type 2 diabetic patients. Impact on microangiopathy

Authors. G.P. Fadini et al.

ELECTRONIC SUPPLEMENTARY MATERIAL

SUPPLEMENTAL TABLES

ESM Table 1. Multivariate analyses.

Variable	M2 percentage	
	Beta	**P**
Type 2 diabetes	-0.401	0.029
Age	-0.197	0.524
Sex male	-0.087	0.439
BMI	0.179	0.563
HbA_{1c}	0.150	0.690
Hypertension	-0.021	0.867
Total cholesterol	-0.160	0.837
HDL cholesterol	0.082	0.754
LDL cholesterol	-0.015	0.979
Retinopathy	0.036	0.717
Chronic renal failure	-0.018	0.858
Atherosclerosis	-0.074	0.512

ESM table 2. Characteristics of patients with available bone marrow samples. *p<0.05 versus controls.

Characteristic	**Control subjects**	**Diabetic patients**
Number	5	5
Age, years	66.8±1.1	69.0±4.2
Sex male, %	100	100
Smoking habit, %	20	0
Hypertension, %	80	100
Dyslipidemia, %	60	60
BMI, kg/m^2	27.2±2.6	30.8±1.4
Fasting glucose, mg/dL	93.2±4.4	229.8±60.4*
Total cholesterol, mg/dL	182.6±8.0	152.8±18.6
HDL cholesterol, mg/dL	51.0±8.1	40.0±2.1
Triglycerides, mg/dL	180.8±95.9	194.4±32.4
LDL-cholesterol, mg/dL	96.0±20.6	74.2±14.3
Ischemic cardiomyopathy, %	60	80

ESM table 3. Characteristics of patients undergoing bone marrow stimulation. *p<0.05 versus controls.

Characteristics	Control subjects	Diabetic patients
Number	14	13
Age, years	40.1±3.8	57.5±1.7*
Sex male, %	78.6	84.6
BMI, kg/m^2	25.8±1.3	29.6±2.1
Hypertension, %	14.3	100.0*
Retinopathy, %	0.0	23.1
Microalbuminuria, %	0.0	7.7
Neuropathy, %	0.0	23.1
Macroangiopathy, %	0.0	46.2*
Insulin, %	0.0	76.9*
Oral agents, %	0.0	69.2*
Statins, %	0.0	76.9*
ACE-inhibitors, %	7.1	76.9*
Other anti-hypertensives, %	0.0	53.8*

ESM References

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2. Wong KL, Tai JJ, Wong WC et al (2011) Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood 118:e16-31

SUPPLEMENTAL FIGURES

ESM Figure 1. Characterization of M1 and M2 monocytes.

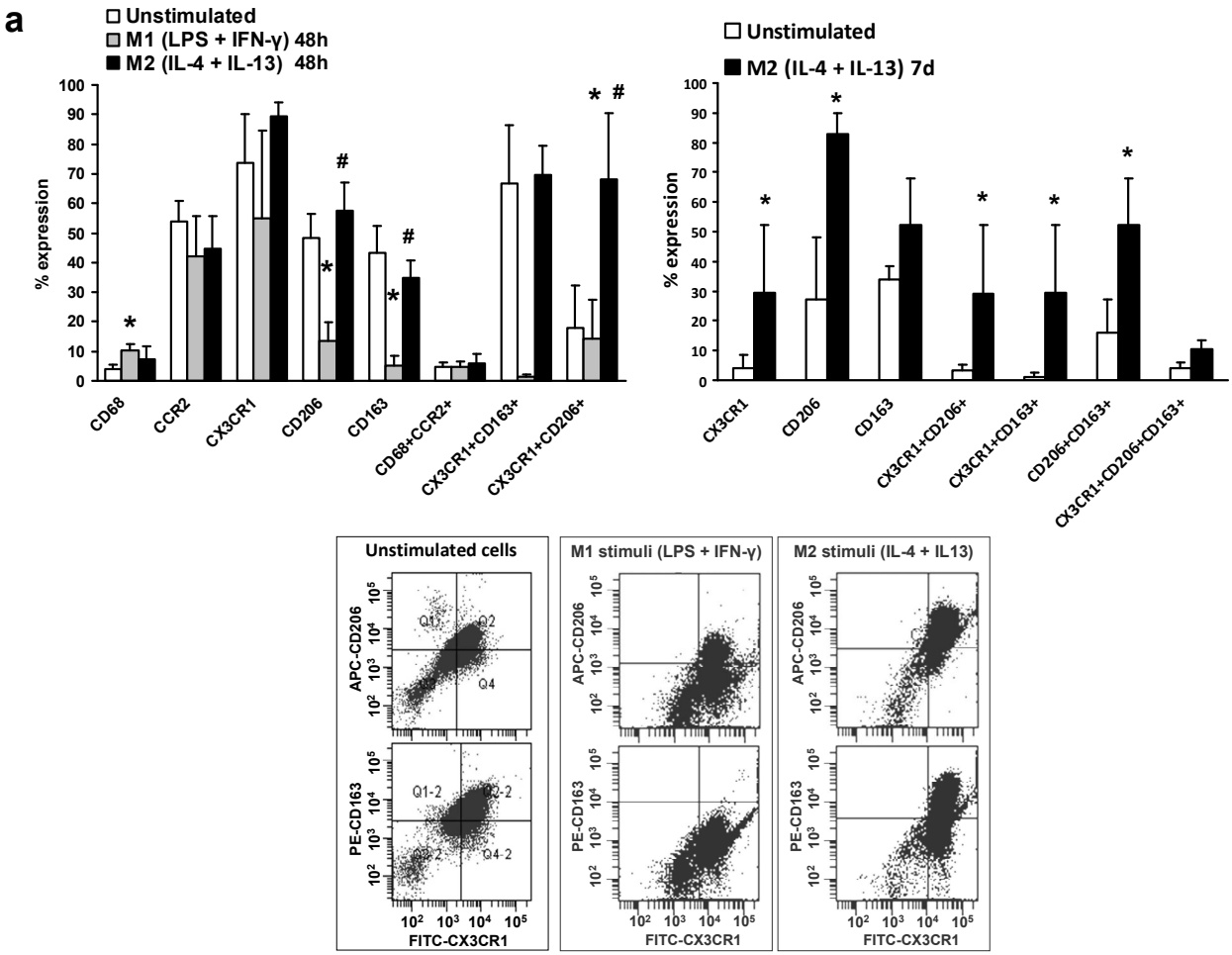
a) Comparison of M1 and M2 monocyte markers with in vitro polarized macrophages and unstimulated cells. The experiments were performed with M1 and M2 stimulation for 48h and with M2 stimulation for 7 days. Representative data of 3-5 independent experiments. *$p<0.05$ vs unstimulated cells; $^{\#}p<0.05$ M2 vs M1. FACS plots are representative of differentially M2 markers in unstimulated cells and macrophages polarized to M1 or M2 for 48h.

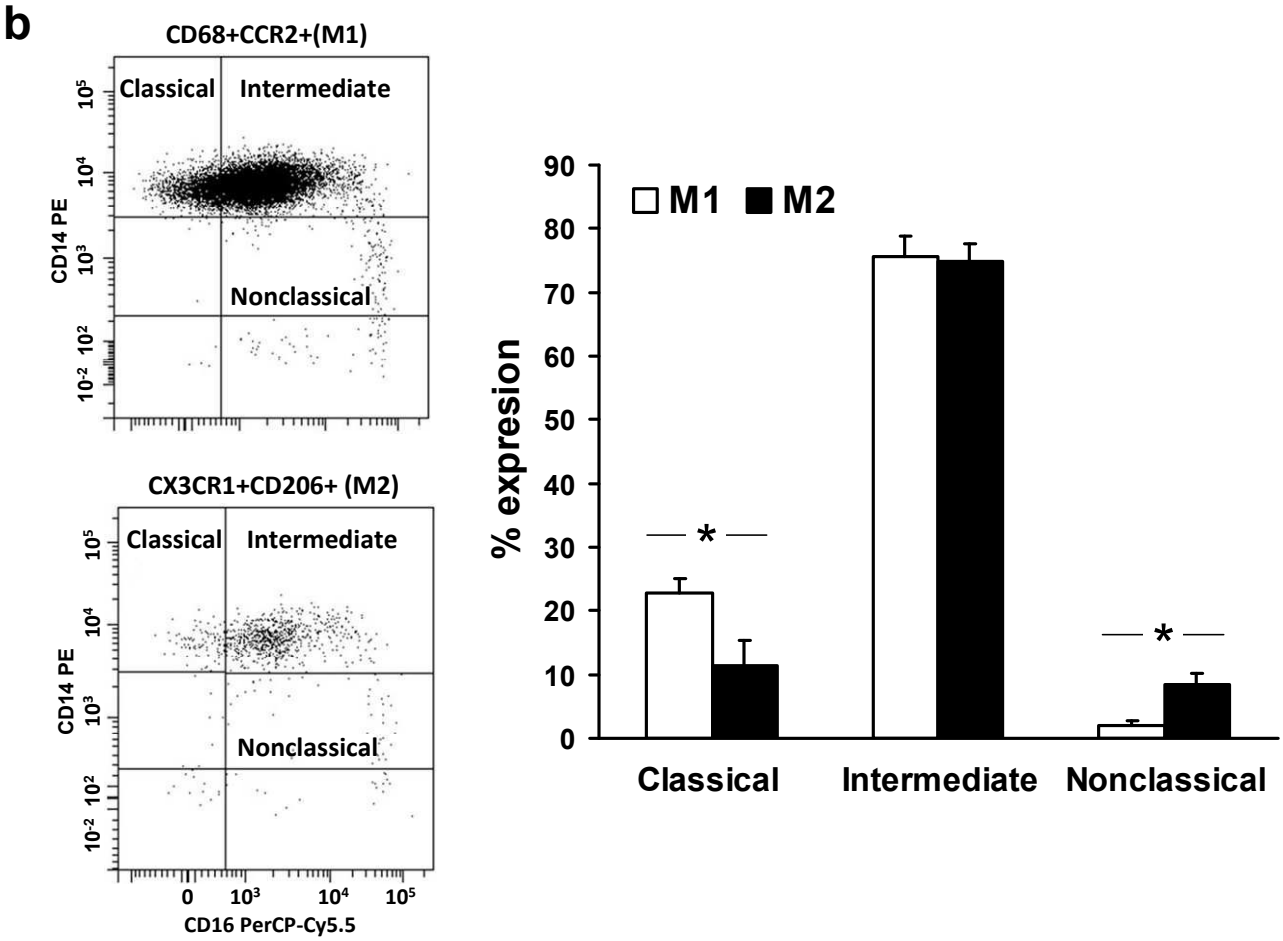
b) The analysis of the expression of CD14 and CD16 on M1 and M2 monocytes allows their definition according to traditional monocyte subsets (classical, intermediate and nonclassical). The percentages of M1 and M2 cells in each quadrant of the CD14/CD16 plot have been compared. Representative data of 5 independent experiments. *$p<0.05$ M1 vs M2.

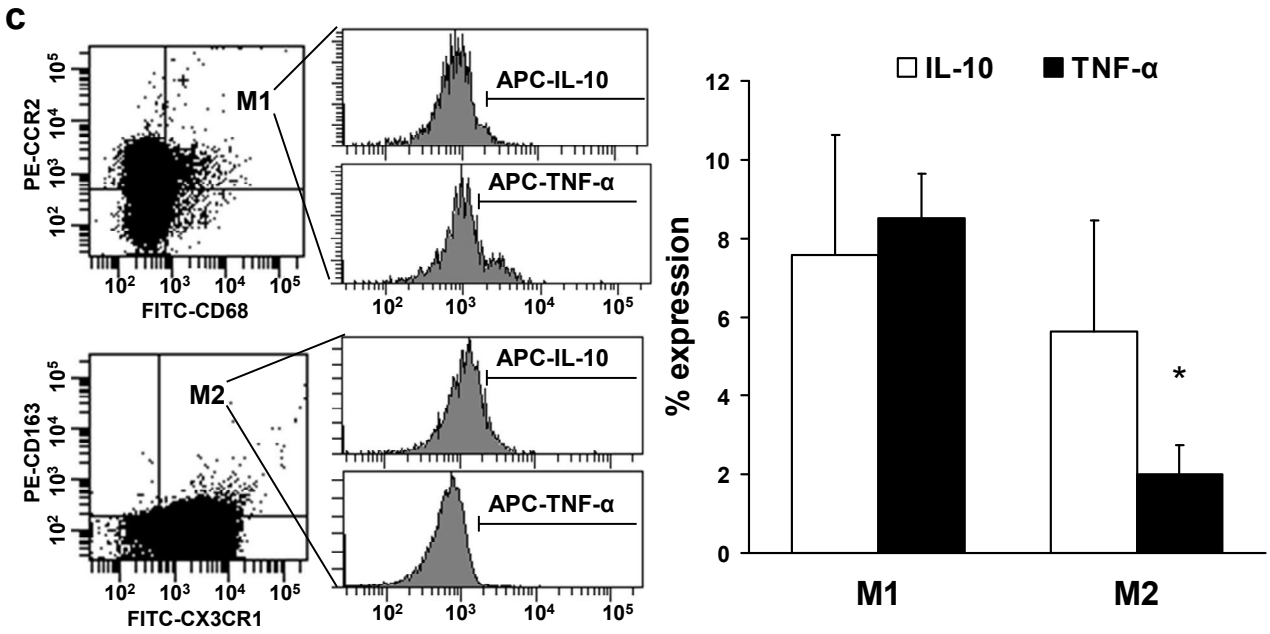
c) Cytokine expression in M1 and M2 cells. Intracellular staining for TNF-alpha or IL-10 (APC fluorescence) has been analysed by FACS in gated M1 (CD68+CCR2+) or M2 (CX3CR1+CD163+ cells). Representative data of 5 independent experiments. *$p<0.05$ M2 vs M1.

d) Uptake of acetylated LDL by M1 (CD68+CCR2+) and M2 (CX3CR1+CD206+) cells was analyzed by FACS. Representative data of 5 independent experiments. *$p<0.05$ M2 vs M1.

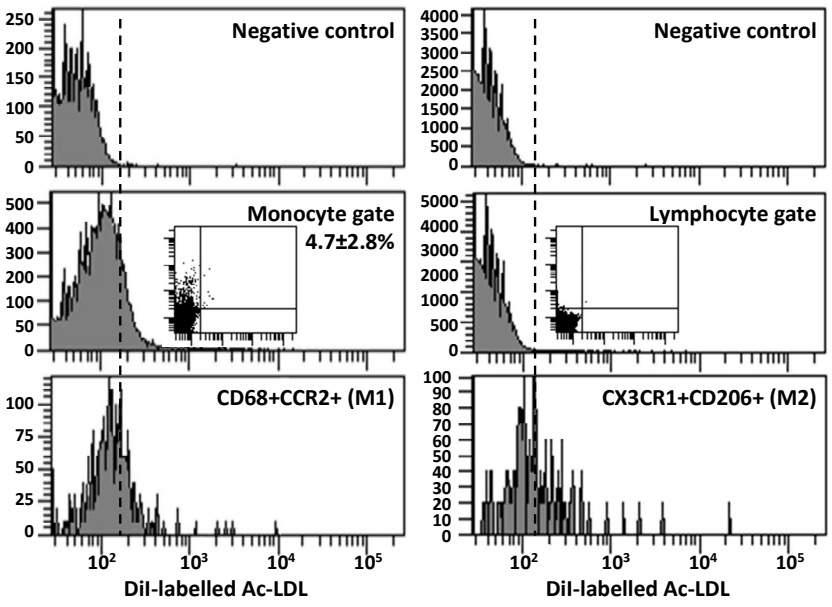
f) Transendothelial migration of M1 (CD68+CCR2+) and M2 (CX3CR1+CD206+) cells in the modified transwell system. FACS scatter plot are representative examples of spontaneous migration. Representative data of 3 independent experiments. *$p<0.05$ M1 vs M2.

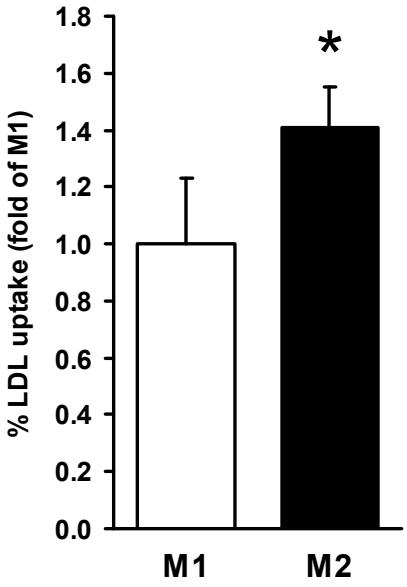

a
Unstimulated
M1 (LPS + IFN-γ) 48h
M2 (IL-4 + IL-13) 48h
% expression
CD68
CCR2
CX3CR1
CD206
CD163
CD68+CCR2+
CX3CR1+CD163+
CX3CR1+CD206+
Unstimulated
M2 (IL-4 + IL-13) 7d
% expression
CX3CR1
CD206
CD163
CX3CR1+CD206+
CX3CR1+CD163+
CD206+CD163+
CX3CR1+CD206+CD163+
Unstimulated cells
M1 stimuli (LPS + IFN-γ)
M2 stimuli (IL-4 + IL13)
APC-CD206
PE-CD163
FITC-CX3CR1
Q1
Q2
Q4
Q1-2
Q2-2
Q4-2

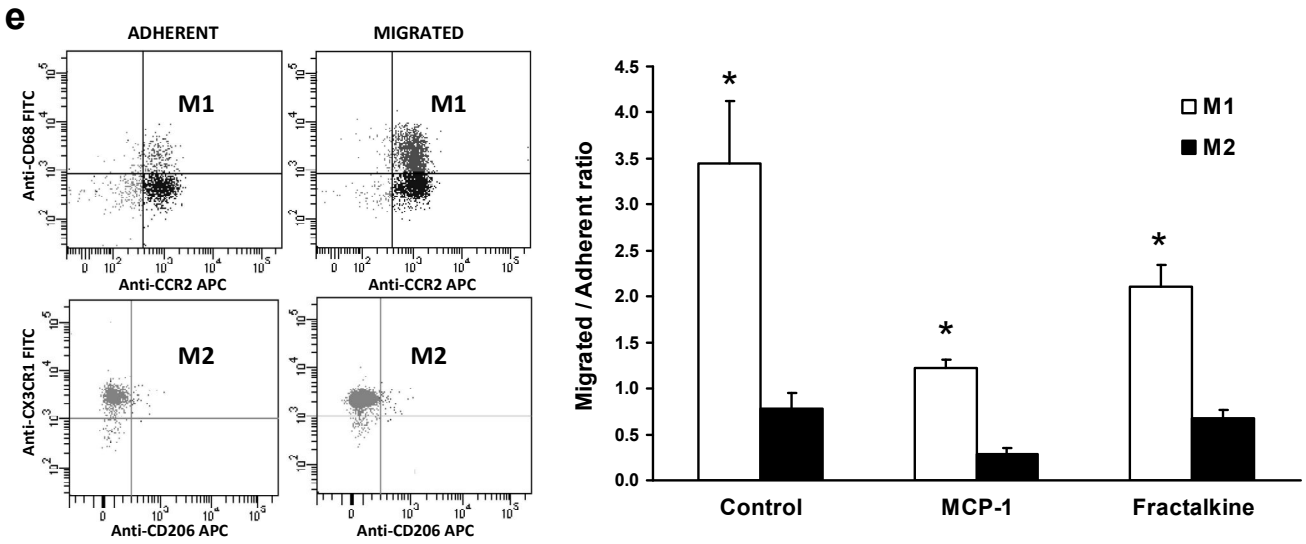

b
CD68+CCR2+(M1)
Classical
Intermediate
Nonclassical
CD14 PE
CX3CR1+CD206+ (M2)
Classical
Intermediate
Nonclassical
CD14 PE
CD16 PerCP-Cy5.5
M1
M2
% expresion
Classical
Intermediate
Nonclassical
*
*


c
PE-CCR2
FITC-CD68
M1
APC-IL-10
APC-TNF-α
PE-CD163
FITC-CX3CR1
M2
APC-IL-10
APC-TNF-α
IL-10
TNF-α
% expression
M1
M2
*

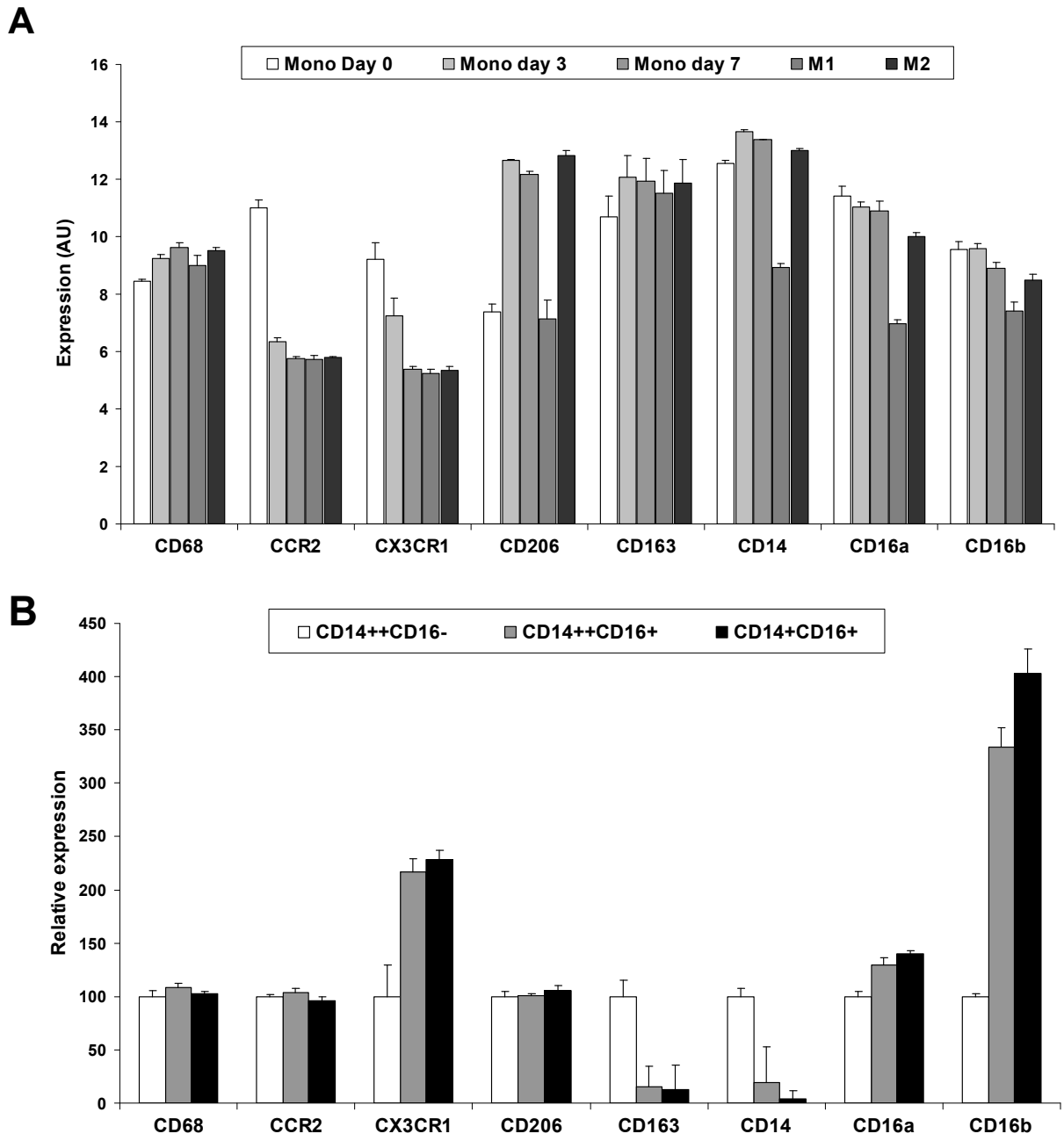
d


Negative control
Negative control
Monocyte gate
4.7±2.8%
Lymphocyte gate
CD68+CCR2+ (M1)
CX3CR1+CD206+ (M2)
DiI-labelled Ac-LDL
DiI-labelled Ac-LDL


% LDL uptake (fold of M1)
*
M1
M2


e
ADHERENT
MIGRATED
M1
M1
M2
M2
Anti-CD68 FITC
Anti-CCR2 APC
Anti-CCR2 APC
Anti-CX3CR1 FITC
Anti-CD206 APC
Anti-CD206 APC
Migrated / Adherent ratio
M1
M2
Control
MCP-1
Fractalkine

ESM Figure 2. In silico gene expression analysis of selected M1 and M2 markers. A) Gene expression of M1 and M2 markers in samples of cells during the monocyte-to-macrophage differentiation and polarization. Data are derived from [1]. B) Gene expression of M1 and M2 markers in traditional monocyte subsets defined by CD14 and CD16 expression. Data are derived from [2].



ESM Figure 3. Direct effects of G-CSF on M1/M2 cells. A) Expression of the G-CSF receptor CD114 was identical in circulating M1 and M2 monocytes (n=3 independent experiments). B) Incubation of peripheral blood mononuclear cells with 20 ng/mL G-CSF did not significantly affect M1 and M2 polarization. A modest non significant increase in M1/M2 ratio was found in control subjects (n=5 experimental replicates).